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# IMMUNOGENIC CHARACTERIZATION OF THE DENUE VIRUS SPECIFIED NONSTRUCTURAL GLYCOPROTEIN GP48 (NV3, SOLUBLE COMPLEMENT FIXING ANTIGEN)

Midterm Report

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Efforts through the midterm funding period have been concerned with mechanisms of the protective immune response to the flavivirus nonstructural protein NS1 and identification of protective NS1 domains for incorporation in possible future subunit flavivirus vaccines.

We have reported that immunization with yellow fever (YF) NS1 protects mice and monkeys against lethal YF infection and that mice immunized with dengue (DEN)-2 NS1 are similarly protected against this flavivirus. These observations have since been confirmed and extended - immunization of mice with a YF NS1 recombinant fusion protein conferred partial protection against the virus (Cane & Gould, 1988) and most recently C.J. Lai and coworkers at NIH have demonstrated solid protection after lethal DEN 4 challenge in mice actively immunized with recombinant DEN 4 NS1 alone or in the form of a

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(19 cont.). polyprotein, produced in a baculovirus vector. Vaccination studies with these recombinants in rhesus monkeys are in progress: to date we detected cytolytic activity in all animals given these NS1 (but not E) constructs. In collaboration with Drs. Robert Putnak (WRAIR, Washington, D.C.) and Mark Cochran (MicroGeneSys, West Haven, CN) we characterized the immune response in rabbits to recombinant DEN 2 NS1 produced by similar methods in a baculovirus vector. Results showed a relatively sluggish low titered antibody response compared to that achieved by vaccination with authentic NS1. Moreover, unlike results with Lai's DEN 4 NS1 recombinants, no cytolytic or complement-fixing activity resulted from immunization with the MicroGeneSys NS1 material. The immune response to reduced and alkylated authentic DEN 2 NS1 was also examined in order to get some idea of the extent of denaturation, e.g., conformational change, that would still allow generation of a cytolytic (and therefore presumably protective) immune response - we showed that all twelve cysteine residues of NS1 are involved in intramolecular bonding. Sera against reduced/alkylated NS1 exhibited marginal CF activity but no cytolytic activity. Difficulties inherent in the isolation and purification of adequate amounts of cell culture-derived authentic NS1 encourage us to pursue the recombinant approach as a resource for flaviviral NS1. It is hoped that such material will be forthcoming from work in progress in the laboratories of Drs. Putnak (YF, DEN 2), Henchal (DEN 3) and Lai (DEN 4).

Cumulative data from assays which measure the capacity of anti-NS1 monoclonal antibodies (Mab) to sensitize cells to complement-mediated lysis suggests a correlation between such activity and Mab protective capacity. Results of NS1 epitope mapping experiments using biotinylated anti-YF NS1 Mab and rabbit antisera produced against authentic and recombinant 17D YF NS1 (the latter prepared by Dr. Charles Rice) suggest that complement-mediated lysis is subserved by selected NS1 domains and that the capacity of an antibody to sensitize YF infected cells to complement-mediated lysis depends on both its isotype and epitope specificity.

Attempts to identify NS1 protective epitopes after chemical fragmentation or enzymatic digestion have met with little success. YF NS1 fragments produced by cyanogen bromide or N-Chlorosuccinimide fragmentation failed to react with anti-NS1 Mab by immunoprecipitation or Western blot assay. However, several partial tryptic peptides of DEN 2 NS1 separated by HPLC appeared to react with two monoclonal antibodies, one of which is protective. Unavoidably high peptide loss during collection will require substantially more NS1 protein than available from current sources to pursue this approach. It is hoped that the future availability of recombinant NS1 will provide the necessary material. Using a commercially available kit (PEPSCAN, Cambridge Research Biochemicals, Cambridge, UK), we synthesized all 404 possible overlapping hexapeptides of 17D YF NS1 as well as a 98 amino acid segment of DEN 2 NS1, shown by R. Putnak to be Mab-reactive. Screening of these hexapeptides with all available anti-YF or DEN 2 NS1 Mab failed to identify any reactive hexapeptides. Taken together, the data may suggest that protective epitopes of NS1 are discontinuous and conformationally dependent.

Immune recognition of viral antigen on the surface of infected cells may provide a mechanism of host defense and recovery in flavivirus infection. We used Mab and monospecific polyclonal sera against YF NS1 and virion envelope protein (E) as probes to detect these antigens on the surface of YF-infected

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(19 cont). mouse neuroblastoma (Neuro 2a) and human adenocarcinoma (SW13). Surface NS1 and E were detected on these cells by radiobinding and immunofluorescent assays. Of great interest is the observation that monospecific rabbit anti-NS1 serum, but not monospecific rabbit anti-E serum or anti-E Mab, sensitized YF-infected Neuro 2a and SW13 cells to complement-mediated lysis. Productive 17D YF infection was reduced up to 1000-fold in synchronously infected Neuro 2a maintained in medium containing an $IgG_{2a}$ anti-YF NS1 Mab (1A5) or monospecific rabbit anti-NS1 serum in the presence, but not absence, of complement. That some anti-NS1 Mab interfere with the in vitro cytolytic activity of protective anti-NS1 Mab raises the possibility of undesirable antigen modulation. This will be tested by Mab mixing experiments in the passive immunization YF mouse challenge model. These results suggest a mechanism of protection against flavivirus infection in addition to that provided by direct virus neutralization.

In anticipation of studies to test the protective capacity of a mixed NS1/E subunit preparation in mice, we purified 17D YF E by immunoaffinity chromatography, using a flavigroup-reactive Mab as ligand. Purified material was identified as a 33 Kd protein. Rabbits immunized with E33 produced neutralizing antibody to 17D YF and DEN 2 in high titer and mice immunized with this protein were protected against lethal YF challenge.

Future efforts will focus on the mechanisms of antibody recognition of plasma membrane NS1 and complement activation. Isolation and characterization by sequencing, virulence testing, etc., of YF escape variants resistant to protective anti-NS1 antibody may provide important information in this regard. Similarly, the observation that some anti-NS1 Mab interfere with the lytic activity of protective anti-NS1 antibody by means other than competitive binding will be explored further.

Adequate amounts of authentic DEN 2 NS1 have been accumulated for challenge studies in rhesus monkeys in collaboration with Dr. Bruce Innis (AFRIMS). It is hoped that satisfactory recombinant DEN 2 NS1 will soon be available for parallel study.

FOREWORD

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## INTRODUCTION

We have shown that passive transfer of monoclonal antibodies against the yellow fever and dengue virus nonstructural protein NS1 protects mice against lethal challenge with the respective viruses. Active immunization with purified NS1 conferred protection against lethal infection in rhesus monkeys (yellow fever) and mice (dengue encephalitis). Most recently, protective immunization of mice with recombinant yellow fever or dengue virus NS1 has been demonstrated by others.

### Evidence of a protective immune response to flavivirus protein NS1. Overview.

We have presented evidence of protection in experimental animals against lethal YF and DEN 2 infection by passive transfer of monoclonal antibodies (Mab) against the flavivirus nonstructural protein NS1 and by active immunization with NS1 isolated from YF or DEN 2-infected cells (1,2,3). Although the mechanisms of such protection is presently unclear, cumulative data from our laboratory suggests a correlation between protection and the capacity of anti-NS1 antibody to sensitize flavivirus-infected cells to complement-mediated cytolysis. These results have now been confirmed and extended. Gould and coworkers demonstrated the protective capacity of anti-NS1 YF Mab (4) and Henchal and coworkers (5) obtained similar results with a panel of anti-DEN 2 NS1 Mab. Most recently, Cane and Gould (6) showed that mice immunized with a YF NS1 recombinant expressed in E. coli as a galactosidase fusion protein were partially protected against lethal challenge with the virus and C.J. Lai's group (NIH) has prepared immunogenic baculovirus recombinants expressing DEN 4 gene segments encoding C-prM-E-NS1-ns2a as well as E and NSI individually (7). Mice immunized with each of these constructs were completely protected against lethal intracerebral challenge with DEN 4 (7, C.J. Lai, personal communication). In anticipation of challenge studies, monkeys have been vaccinated with these constructs and preliminary assays in our laboratory show cytolytic activity in sera from animals immunized with the NS1 constructs (see below). Taken together, the NS1 data provide evidence of an alternative to direct viral neutralization in the protective immune response against flavivirus infection.

**Characterization of monoclonal anti-NS1 Mab.**

Table 1 summarizes relevant characteristics of available anti-YF and DEN 2 NS1 Mab produced in our laboratory or in those of our collaborators. More detailed descriptions of these antibodies have been published or are in press (1,4,5).

**Identification of protective NS1 epitopes.**

A. Role of complement-mediated lysis in the protective immune response to NS1.

Cumulative data obtained in the course of the present study strongly suggest a correlation between the protective capacity of anti-NS1 Mab and their capacity to sensitize cells to complement-mediated lysis. Figures 1 and 2 compare lytic activities (in Neuro 2a or SW13 cells, respectively), and protective capacities among available anti-YF NS1 Mab. In general, at least three categories of anti-NS1 Mab activity emerge from these assay results: lytic protective, non-lytic protective, and non-lytic non-protective. That some non-lytic anti-NS1 Mab are protective (Gould's Mab 492, 863, 925, 428, and Henchal's IgG1 Mab) suggests that other mechanisms of antibody-dependent immunity may be operating. Among these, antibody-dependent cell-mediated cytotoxicity (ADCC) would be expected but, to date, we have been unable to demonstrate ADCC using these antibodies in assays employing primary mouse macrophages or a continuous mouse macrophage cell line ($P388D_1$) as effectors and YF or DEN-infected Neuro 2a or SW13 cells as targets.

The capacity of anti-NS1 antibody to sensitize flavivirus-infected cells to complement-mediated lysis indicates that this protein is expressed on the cell surface (discussed further below). As such, recognition by effector arms of the cellular immune response, e.g., cytotoxic T cells (Tc), might be expected. Recent reports of H-2 restricted T cell killing of flavivirus infected targets are supportive in this regard, although antigen specificity of the Tc cells was not determined (8) and only T helper cells were reported after T cell cloning (9).

B. NS1 epitope mapping.

It is presently unclear whether complement-mediated cytolytic activity is subserved by specific NS1 domains or whether such activity is simply determined by an anti-NS1 antibody's complement-fixing capacity without regard to the NS1 domain recognized. Preliminary data from our laboratory is inconclusive. Results of competitive binding assays employing biotinylated anti-YF NS1 Mab are shown in Table 2. This assay measures the capacity of protective and nonprotective anti-YF NS1 Mab to block binding of biotin-labeled

complement-fixing, protective anti-NS1 Mab. Binding avidities among this panel of Mab were quite similar (data not shown). Although the assay was limited by denaturation of the majority of the Mab after biotinylation, a number of conclusions are suggested by these data:

1) At least 2 separate epitopes are recognized by protective cytolytic Mab; 2) a number of nonprotective Mab (4E3, 2G2) recognize a potentially "protective" site(s); 3) two protective antibodies (428, 925) which are not lytic, e.g., complement-mediated, appear to define an epitope distant from at least one which subserves complement-mediated lysis; 4) two antibodies (428, 492) of an isotype ($IgG_{2a}$) expected to fix complement do not compete with the cytolytic Mab and are not cytolytic. Of interest in regard to the last is failure of rabbit serum prepared against recombinant YF NS1 (provided by Dr. Charles Rice) to sensitize cells to complement- mediated lysis or interfere with binding to NS1 of biotinylated cytolytic protective Mab above (1A5, 8G4, 423, 871). The recombinant YF NS1 was constructed as a Tryp E fusion protein and differs from authentic NS1 by deletion of the first 91 amino acids from the NS1 amino terminus. In contrast, rabbit serum that we prepared to authentic YF NS1 was cytolytic and competed with the protective lytic Mab (Figure 3). Both rabbit anti-YF NS1 sera bind to authentic NS1 (the anti-recombinant NS1 less so, Figure 4), immunoprecipitate NS1, and stain YF-infected SW13 and Vero cells in an indirect immunofluorescent assay. Taken together, these results are consistent with the view that complement-mediated lysis is subserved by selected NS1 domains and that the capacity of an antibody to sensitize YF-infected cells to complement- mediated lysis depends both on its isotype and specificity. It is tempting to speculate that the cytolytic domain(s) are located at the NS1 amino terminus, but masking of other NS1 regions by the Tryp E fusion protein could also account for the results.

C. Peptide mapping.

Our approach to identification of NS1 immunoreactive domains has largely assumed that at least some are represented by continuous epitopes or that, if discontinuous, such regions are close enough to each other to allow preservation of antibody recognition after fragmentation of the complete protein. Methods used for fragmentation are completely empirical and have included chemical cleavage with cyanogen bromide (CnBr, methionyl peptide) and N-chlorosuccinimide (NCS), tryptophanyl peptide) or enzymatic digestion with trypsin (lysinyl, argininyl peptide) (10,11, 12). NS1 for this purpose was purified by immunoaffinity chromatography by methods reported earlier from our laboratory (1-3). Initially, immunoaffinity-purified radioiodinated YF and DEN 2 NS1 were subjected to CnBr fragmentation followed by immunoprecipitation with representative

anti-NS1 Mab or monospecific antisera. None of the fragments generated were precipitated. The method was modified by exposing NS1-containing acrylamide gel slices to CnBr vapors (10) in an effort to minimize formic acid-induced denaturation. The resulting fragments were electro-eluted into SDS-acrylamide gels followed by transfer to nitrocellulose paper and immunoblotting. Again, no reactivity was detected with any of the available monoclonal or monospecific anti-NS1 probes. More recently we have found markedly heightened sensitivity of detection of intact NS1 using polyvinylidene difluoride membranes (Immobilon, Millipore) in Western blots, but use of this support also failed to detect NS1 fragments (CnBr, NCS, tryptic) reactive with any of the available anti-NS1 antibodies.

In parallel experiments, we have purified DEN 2 NS1 by reverse-phase HPLC (Figure 5) and generated reproducible tryptic peptide maps. Several peptides appeared to react with two anti-NS1 Mab, one of which is protective (Figure 6). However, these results were inconsistent probably due in large measure to the small amounts of available NS1 and the marginal sensitivity of the immunoassays at the NS1 concentrations used. Additionally, protein retrieval from collection tubes after acetonitrile evaporation was complicated by substantial and apparently irreversible binding to the plastic or glass surface leading to substantial loss. This problem has been commented upon by others and represents an example of the highly empirical nature of this methodology (14).

The approach of Geysen et al. (15) was used to systematically synthesize all possible short (hexa-) peptides of YF as well as part of DEN 2 using sequences provided by Drs. Charles Rice, Vincent Deubel, and Robert Putnak. This computer-assisted method allowed rapid concurrent synthesis and immunoassay (ELISA) of all 404 possible overlapping hexapeptides covering the total 409 amino acid sequence of 17D YF NS1. Results were disappointing: screening of YF NS1 hexapeptides with each available anti-YF Mab or monospecific rabbit and monkey (2) anti-YF NS1 sera failed to demonstrate a single convincingly positive peptide on multiple tests. Similarly, no reactivity with any DEN 2 NS1 Mab was detected against amino acid residues 261-359 of DEN NS1 shown by R. Putnak to be reactive with several Mab as well as monospecific rabbit anti-NS1 sera in a Western blot analysis of recombinant NS1 produced in E. Coli. Correct synthesis of control peptides included in the "PEPSCAN" kits suggests that our failure to detect reactive NS1 peptides was not the result of error in synthesis. We conclude that possible continuous NS1 epitopes recognized by these antibodies are likely to be larger than 6-mers. The expense and uncertainty of this approach led us to abandon it. Dr. Lew Markoff (NIH) is presently studying overlapping 15-20 mers of DEN 4 NS1 for immunoreactivity (personal communication) and his

results are awaited with great interest. However, taken together our results strongly suggest that the available protective YF and DEN 2 NS1 Mab are directed at discontinuous and probably conformationally determined epitopes (see below).

D. Immunoreactivity/immunogenicity of recombinant DEN 2 and 4 NS1.

A problem central to the NS1 fragmentation work has been our inability to isolate NS1 in large enough quantity. Recombinant DEN 2 NS1 (New Guinea C) prepared in a baculovirus vector by MicroGeneSys (West Haven, CN) and partially purified by them was made available to us for further characterization. It was hoped that this material would resolve the NS1 supply problem. In preliminary experiments we compared the anti-NS1 immune responses among rabbits immunized with recombinant and authentic DEN 2 NS1. Additionally, the immune response to reduced and alkylated authentic DEN 2 NS1 was examined in order to get some idea of the extent of denaturation, e.g., conformational change, that would still allow generation of a cytolytic (and therefore presumably protective) immune response: all (twelve) cysteine residues are involved in intramolecular bonding (Figure 7). Figure 8 shows the antibody response against **authentic** NS1 among rabbits immunized with either authentic or recombinant NS1 or reduced and alkylated authentic NS1. Antibody titers were measured in an ELISA incorporating affinity-purified authentic DEN 2 NS1 in the solid phase. A brisk antibody response was seen among rabbits immunized with native or denatured authentic NS1. However, the response was delayed and of low titer among rabbits immunized with the MicroGeneSys recombinant material. When these sera were tested in parallel against <u>recombinant</u> NS1 (Figure 9), similar high titers developed in rabbits immunized with authentic NS1 and to a lesser degree after immunization with reduced and alkylated NS1. One rabbit immunized with recombinant NS1 developed substantial titers by 8 weeks, whereas titers in the other remained on the low side. These results are reflected by Western blot analysis shown in Figures 10 and 11. The figures show blot results at the time of the first and second boost and last bleed at 3,6, and 8 weeks post-prime, respectively. Rabbits immunized with authentic NS1 generated strong specific antibody responses to the 46 and 44 Kd moieties of NS1, as did the rabbit immunized with reduced and alkylated NS1. Only weak responsiveness was seen by 8 weeks in the two rabbits immunized with the recombinant NS1 preparation. Similarly, sera from rabbits immunized with authentic NS1 developed antibody against recombinant NS1, as shown by Western blots in Figure 11. Of note is the somewhat higher molecular weight of recombinant NS1 (ca. 52-55 Kd) and its appearance as a triplet which probably reflects glycosylation differences. A consistent positive blotting "smudge" appears at the top of the recombinant

NS1 strips, which we believe reflects polymerization of the recombinant protein. This is a consistent finding in our hands. Mab prepared to authentic DEN NS1 did not react with the recombinant protein in this assay. Of potential importance is the fact that cytolytic antibody developed only in rabbits immunized with authentic NS1: sera from rabbits immunized with reduced and alkylated authentic NS1 or recombinant NS1 were not lytic. Similarly, substantial complement-fixing activity was present only in sera from authentic NS1-immune rabbits (Table 3). These results, though preliminary, are somewhat disappointing, if the proposition that cytolytic activity correlates with protective capacity is correct: critical changes in the conformation of candidate small NS1 peptide vaccines may be required if the immune response to them is to be protective.

Preliminary results with sera from rabbits and monkeys immunized with DEN 4 baculovirus recombinants from C.J. Lai's laboratory have been more encouraging. Both rabbit and monkey sera against DEN 4 constructs consisting of NS1 alone, or as part of a C$\longrightarrow$NS1 polyprotein, sensitized DEN 4-infected cells to C'-mediated cytolysis (Table 4) and these constructs (as well as that of E) protected mice against lethal challenge (C.J. Lai, personal communication). Results of DEN 4 challenge among monkeys immunized with these recombinants should be available shortly.

**Cell surface expression of NS1.**

Current knowledge of flavivirus maturation and release suggests that exit of these viruses from infected cells is largely by exocytosis of virion-containing vesicles (16), although evidence of budding from the plasma membrane has also been presented (17). That anti-YF NS1 Mab sensitize YF-infected cells to complement-mediated lysis provides strong evidence that NS1 is cell membrane associated. Table 5 examines the cytolytic activities of monospecific rabbit sera prepared to YF NS1 and E using YF-infected Neuro 2a and SW13 target cells. Rabbit sera against the respective uninfected cells served as positive controls. Anti-NS1, but not anti-E, serum was cytolytic as were the respective controls. These data, along with our failure to demonstrate cytolytic activity among 11 anti-YF E Mab (9/11 are $IgG_{2a/2b}$) suggested the absence of detectable plasma membrane E. However, in direct biding assays employing radioiodinated anti-NS1 or E Mab and in indirect binding assays with radioiodinated Staph. protein A and monospecific rabbit antibody against NS1 or E, both proteins were detected on the surface of Neuro 2a and SW13 cells (Table 6). These results are consistent with speculation that NS1 is an integral plasma membrane protein, whereas E is recognized in the form of released virion which accumulates on the cell surface remaining loosely bound. Washed infected cells held at $4^{o}C$ bound substantially less anti-E antibody.

The effect of anti-NS1 antibody (1A5) and complement on the replication of 17D YF in Neuro 2a cells was examined as well (Figure 12). Peak titers of virus were reached by 48 hours at which time there was a greater than 100-fold reduction in titrable virus from cells treated with antibody 1A5 and complement. At 48 hours post infection no CPE was seen under any treatment condition, and fewer than 5% of cells treated with 1A5 and complement were IFA-positive, whereas 50-75% of cells treated with antibody and inactivated complement or myeloma controls were IFA-positive. Monolayers treated with antibody 1A5 and active complement were intact with minimal CPE at 70 hours post infection at which time CPE was complete under all other conditions. In parallel assays, non-complement-fixing anti-NS1 antibodies had no effect on virus production in the presence or absence of complement. Table 7 summarizes results with anti-NS1 Mab and monospecific rabbit serum in both Neuro 2a and SW13 cells. In collaboration with Dr. Robert Putnak (WRAIR) we will attempt to isolate and characterize conditional 17D YF mutants selected by growth in Neuro 2a and SW cells under complement and antibody pressure. NS1 sequencing of such mutants could provide important information about sites critical to complement-mediated cytolysis and, presumably, regions of NS1 that subserve protection.

To our surprise, we have found that selected non-lytic anti-NS1 Mab can substantially interfere or completely abrogate the cytolytic activity of protective anti-NS1 Mab. In some instances this is the case even though the interfering antibody shows no evidence of competition with the protective antibody for binding to purified NS1 or to live infected cells. Figure 13 shows representative results of an experiment comparing the lytic activity of protective anti-YF NS1 antibody 1A5 (serially diluted) in the presence of a single low dilution of control myeloma protein PC5 or non-protective non-cytolytic anti-YF Mab (2D10). In separate assays we determined that these interfering anti-NS1 Mab were not anti-complementary, since they had no effect on the lytic activity of rabbit serum raised against the SW cell membrane itself. That interfering antibodies also reduced the lytic activity of monospecific rabbit serum to NS1 is consistent with the idea that their effect may result from cross-linking and capping of plasma membrane NS1 with subsequent loss of this antigen from the cell surface. The in vivo significance of this observation demands further study and will be addressed in future experiments.

**Preliminary immunogenic characterization of 17D YF envelope protein E.**

In anticipation of studies to test the protective effect of immunization with NS1/E subunit mixtures in mice, we purified 17D YF E from lysates of infected Vero cells by

immunoaffinity chromatography using a flavigroup-reactive Mab as the ligand. Purified material could not be identified as intact E but bore antigenic determinants of E as defined by selective reactivity with anti-E monoclonal antibodies. The reactivity correlated with a 33 Kd band, the predominant component on polyacrylamide gel electrophoresis. Rabbits hyperimmunized with E33 produced neutralizing antibody to YF in titers similar to those obtained with a single dose of 17D YF vaccine (Table 8). The rabbit antisera had a high degree of cross-neutralizing activity against DEN 2, whereas the human antisera, as expected, failed to neutralize dengue. Rabbits immunized with high dose live 17D YF produced higher titers of neutralizing antibody to 17D YF, but cross-neutralizing activity against dengue was lower than that seen in rabbits immunized with E33. This E protein fragment, then, appears to express a group-reactive determinant, suggesting the possibility of heterotypic protection after vaccination.

Mice actively immunized with E33 were protected against lethal intracerebral challenge with YF: 16/20 E33-immune mice survived vs. 6/33 control ovalbumin-immune mice ($p<0.001$).

## CONCLUSION

Cumulative results from our laboratory and from those of our collaborators indicate that the immune response to the flavivirus nonstructural protein NS1 is protective, thereby providing an alternative to direct viral neutralization in the defense against these viruses. That the immune enhancement phenomenon should not be an issue with anti-NS1 antibody further emphasizes the potential value of dengue vaccines incorporating this protein. Cumulative data from monoclonal antibody screening of peptide fragments of yellow fever and dengue NS1, as well as overlapping hexapeptides of this protein, have, in our hands, failed to provide evidence that protective NS1 epitopes are expressed as continuous determinants. To the contrary, it seems highly probable that NS1 recognition by protective antibody is conformationally dependent. Failure of a number of flavivirus E and NS1 recombinants, with anticipated conformational variance, to provide evidence of a protective immune response is consistent with this belief.

Further efforts in our laboratory will focus on the mechanisms of antibody recognition of plasma membrane NS1 and resulting complement activation. In preliminary experiments we have found that selected nonprotective YF monoclonal antibodies can substantially interfere with or entirely abrogate the cytolytic capacity of protective anti-NS1 antibodies. Since the interfering antibodies do

not compete with binding by the cytolytic antibodies, the mechanisms of interference is unclear. One possibility is that they modulate plasma membrane NS1 expression in a manner similar to that described for measles virus (18). Should this be true, then appearance of such antibody could be undesirable, leading to loss of immune recognition of flavivirus-infected cells and persistence of infection. This possibility will be tested in passive immunization experiments. Abrogation of protection conferred by cytolytic monoclonal anti-NS1 antibody in mice concurrently immunized with "interfering" monoclonal anti-NS1 antibody would have obvious implications for the design of NS1 immunogens. The importance of complement activation in the protective response will be further explored by comparing the protective capacities of univalent ($Fab_1$ Fab/c), bivalent ($Fab_2$), and intact IgG NS1 Mab in the YF NS1 mouse model.

In parallel experiments we will attempt to select YF mutants resistant to the effect of complement-mediated cytolytic antibody. Cloning and sequencing of such variants (in collaboration with Dr. Robert Putnak) should provide important information about NS1 epitopes involved in immune cytolysis. In essence, sequencing of such variants may enable us to identify elements of a conformationally determined protective NS1 region which could not be mapped by the fagmentation of "overlapping peptide" appraoches. It is anticipated that such variants would be lethal to mice passively immunized with Mab which would otherwise protect against the parent strain of virus.

Enough chromatographically purified DEN 2 NS1 has now been accumulated to permit evaluation of its protective capacity in rhesus monkeys and this is currently being planned with Dr. Bruce Innis (AFRIMS). This experiment had been delayed in the hope that authentic and recombinant NS1 could be tested simultaneously; failure of the MicroGeneSys material to induce a satisfactory immune response in rabbits has ruled this material out.

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Table 1. Characteristics of anti-NS1 monoclonal antibodies

**DEN 2**

| Clone | Immunizing DEN Strain | Ig | ELISA titer ($-\log_{10}$) vs DEN 2 (NGC) NS1 | Protection |
|---|---|---|---|---|
| *16-25/3 | D80-100 | G1 | < 2 | - |
| *20-1/1 | " | G1 | < 2 | - |
| *27-12/4 | " | G1 | 5.8 | + |
| *34-23 | " | G1 | 4.8 | - |
| *40-21/9 | " | G2b | 5.6 | - |
| *47-10/10 | " | G2a | 2.7 | - |
| *63-15 | " | G1 | 5.6 | + |
| *68-5/16 | " | G1 | 5.3 | + |
| *101-47 | " | G1 | 2.3 | - |
| **6A8 | NGC | M | ND | + |
| **9A9 | " | G1 | > 5.0 | - |
| **4D11 | " | G1 | 3.7 | - |
| +D7-4E9 | " | G1 | > 5.0 | - |

**17DYF**

| Clone | Immunizing YF Strain | Ig | ELISA titer ($-\log_{10}$) vs 17D YF | Protection |
|---|---|---|---|---|
| #863 | Porterfield | G1 | > 7.0 | + |
| #925 | " | G1 | 5.0 | + |
| #871 | " | G2a | 6.0 | + |
| #979 | " | G2a | 3.0 | + |
| #999 | " | G2a | 6.0 | + |
| #993 | " | G2a | 6.0 | + |
| #917 | " | G2a | 6.0 | + |
| #992 | " | G2a | 5.0 | + |
| #423 | " | G2a | 6.0 | + |
| #492 | " | G2a | < 2.0 | + |
| #428 | " | G2a | 5.0 | + |
| #924 | " | M | < 2.0 | + |
| *1A5 | Connaught | G2a | > 7.0 | + |
| *8G4 | " | G2b | 6.0 | + |
| *4E3 | " | G1 | > 7.0 | - |
| *2G2 | " | G3 | ND | - |
| *2D10 | " | G1 | 6.0 | - |

* EA Henchal  + WRAIR  # EA Gould  ** Rochester

Table 2. Competitive Binding Assay

| | Unlabeled competitor anti-YF NS1 monoclonal antibody | Isotype IgG | Biotinylated anti-YF NS1 monoclonal antibody | | | |
|---|---|---|---|---|---|---|
| | | | 1A5 | 8G4 | 423 | 871 |
| | 1A5 | 2a | +[a] | + | + | + |
| | 8G4 | 2b | + | + | + | + |
| | 423 | 2a | - | +/- | + | + |
| Protective, cytolytic | 871 | 2a | + | + | + | + |
| | 992 | 2a | - | - | - | - |
| | 999 | 2a | - | - | - | - |
| | 979 | 2a | - | - | - | - |
| | 917 | 2a | - | - | + | - |
| Protective, not cytolytic | 428 | 2a | - | - | - | - |
| | 925 | 1 | - | - | - | - |
| | 2D10 | 1 | - | - | - | - |
| | 4E3 | 1 | + | + | + | + |
| Not protective not cytolytic | 2G2 | 3 | + | + | + | + |
| | 863 | 1 | - | - | - | - |
| | 492 | 2a | - | - | - | - |

[a] competition + = >75%; +/- = 25-50%; - = <1%

Table 3. Complement fixing activity among sera from rabbits hyperimmunized with authentic, recombinant (MicroGeneSys), or reduced/alkylated DEN 2 NS1

| | | CF titers against DEN serotype | | | | |
|---|---|---|---|---|---|---|
| Rabbit | DEN 2 NS1 | 1 | 2 | 3 | 4 | NMB[a] |
| 1 | Authentic | 128 | 1,024 | 256 | 64 | 64 |
| 2 | Authentic | 512 | 16,384 | 2,048 | 128 | 32 |
| 3 | Recombinant | 16 | 16 | 16 | 16 | 16 |
| 4 | Recombinant | 8 | 8 | 8 | 8 | 16 |
| 5 | Reduced/alkylated | 32 | 64 | 16 | 8 | 16 |

[a] normal mouse brain control

Table 4. Complement-mediated cytolytic activity among rhesus monkeys immunized with recombinant DEN 4 protein

| | | Day post-primary immunization % specific $^{51}$Cr release[a] | | |
|---|---|---|---|---|
| Immunogen | Mk# | Day: 0 | 28 | 35 |
| Control SF9 | 058D | 0 | 0 | 0 |
| | 243 | 0 | 0 | 2 |
| | 373D | 2 | 12 | 3 |
| NS1 | 003D | 0 | 0 | 12 |
| | 3J8 | 0 | 6 | 20 |
| | 4GV | 4 | 1 | 17 |
| C⟶NS1 | 190D | 0 | 6 | 11 |
| | 200D | 3 | 2 | 18 |
| | 819C | 0 | 8 | 29 |
| E | 070D | 0 | 0 | 0 |
| | 4IJ | 3 | 4 | 4 |
| | 927C | 0 | 0 | 0 |

[a] All sera (@1/50) assayed at same time in quadruplicate.

Table 5. Complement-mediated lysis of 17D YF-infected SW13 or Neuro 2a cells

| | % specific $^{51}Cr$ release from 17D infected: | |
|---|---|---|
| Rabbit anti- | SW13 | Neuro 2a |
| YF NS1 | 54 | 16 |
| YF E | 2 | 2 |
| SW13 | 34 | - |
| Neuro 2a | - | 72 |

Table 6. Binding of radioiodinated monoclonal anti-YF NS1 or E antibodies to YF-infected SW13 or Neuro 2a cells

| | SW13 | | Neuro 2a | |
|---|---|---|---|---|
| | 37°C | 4°C | 37°C | 4°C |
| *[a]1A5±PC5 | 2220±214 [b] | 592±106 | 2108±468 | 979±203 |
| *1A5±1A5 | 270±128 | 90±42 | 121±45 | 85±11 |
| *2E10±PC5 | 2100±254 | 404±122 | 365±90 | 203±90 |
| *2E10±2E10 | 256±78 | 77±30 | 96±36 | 98±37 |

[a] *Radioiodinated Mab

[b] cpm bound±sd; quadruplicate samples

Table 7. Growth of 17D YF in tissue culture in the presence of antibody and complement

| Antibody | Complement | Cell type N2a | SW13 |
|---|---|---|---|
| PC5 | -[a] | 6.3[b] | 7.0 |
| | + | 6.2 | 7.1 |
| 1A5 | - | 6.4 | 7.0 |
| | + | 4.1 | 5.3 |
| NRS | - | 6.2 | 7.1 |
| | + | 6.1 | 7.1 |
| R anti-YF NS1 | - | 6.3 | 7.? |
| | + | 5.2 | 6.3 |

[a] Complement (-) = heat inactivated, (+) = active

[b] $Log_{10}$ PFU/ml culture supernatant titered in Vero cells

Table 8. Neutralizing antibody titers in rabbits immunized with 17D YF virion or E p33

| Rabbit immunized with: | PRNT | |
|---|---|---|
| | 17D YF | DEN 2 |
| E p33[a] | | |
| #1 | 256 | 64 |
| 2 | 64 | 64 |
| 3 | 128 | 32 |
| 4 | 512 | 256 |
| 17D virion[b] | | |
| #1 | 1,024 | 32 |
| 2 | 2,048 | 32 |

[a] ca 25ug x 3

[b] $10^6$ PFU I.V.

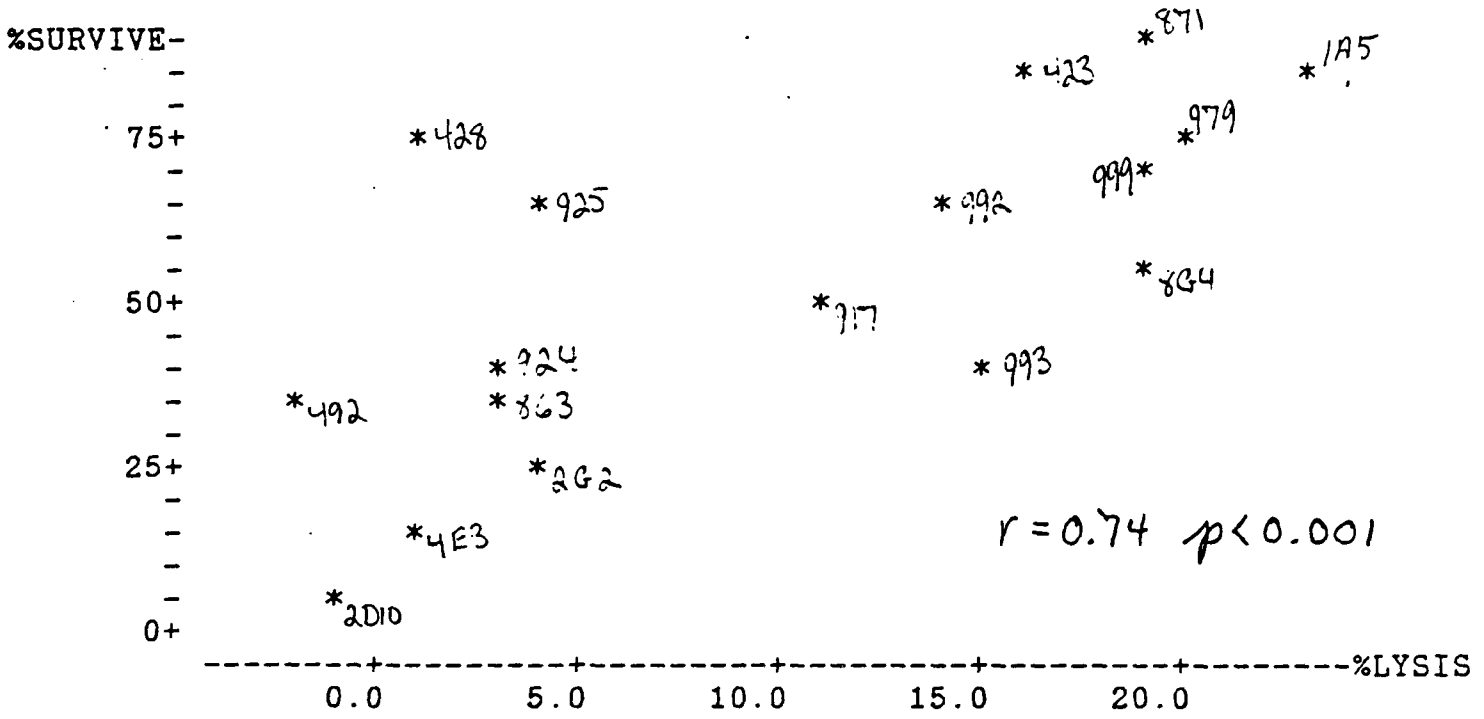


Figure 1. Lytic versus protective capacities among anti-YF NS1 Mab. Mab (Table 1) were ordered by protective capacity as determined by reported (Ref. 1,4) survival in intracerebral mouse protection tests and cytolytic activity measured by per cent specific $^{51}Cr$ release from 17D YF-infected mouse neuroblastoma cells (Neuro 2a).

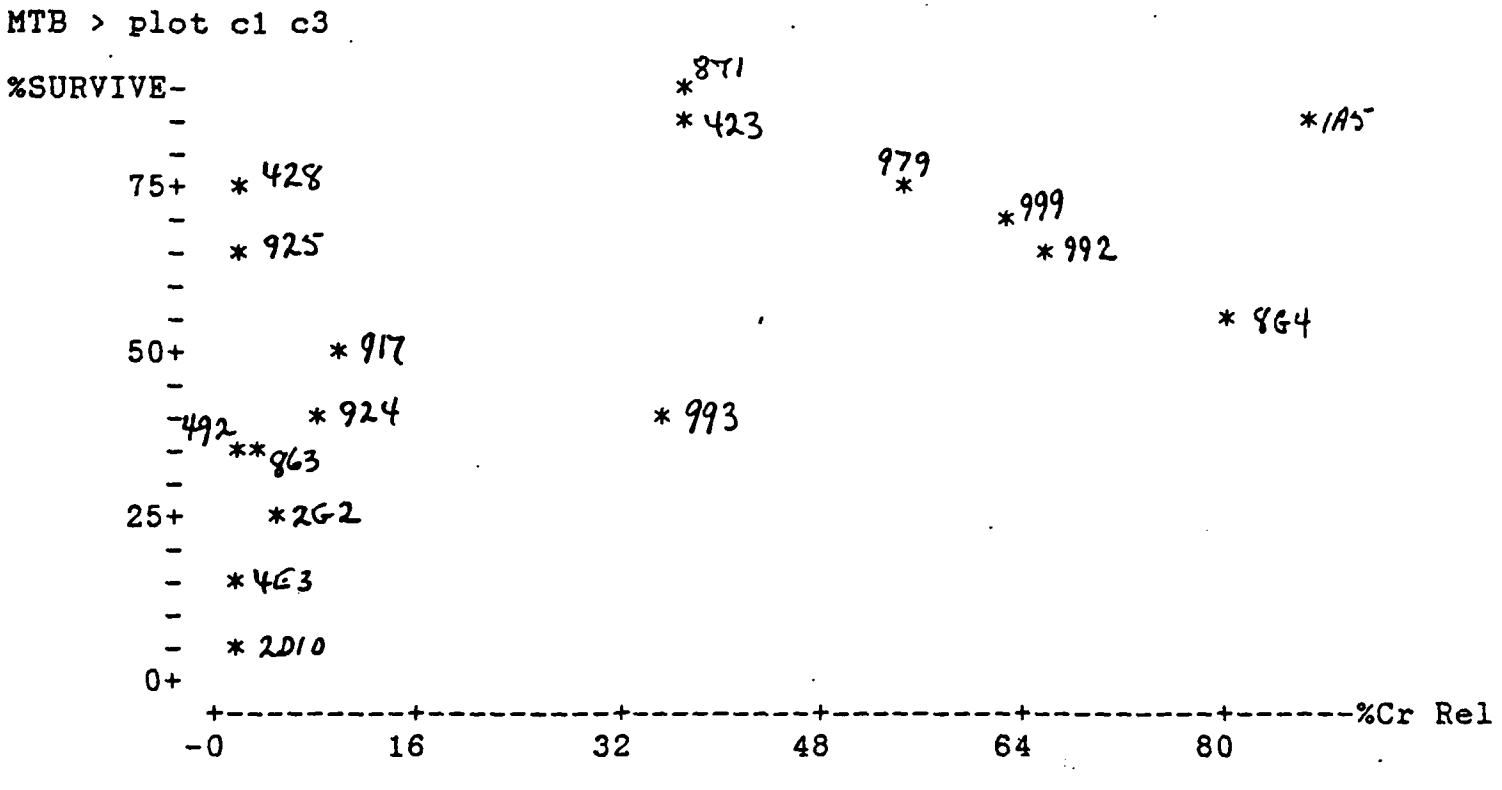


Figure 2. Lytic versus protective capacities among anti-YF NS1 Mab. As in Figure 1, but % lysis determined in 17D YF-infected human adenocarcinoma cells (SW 13).

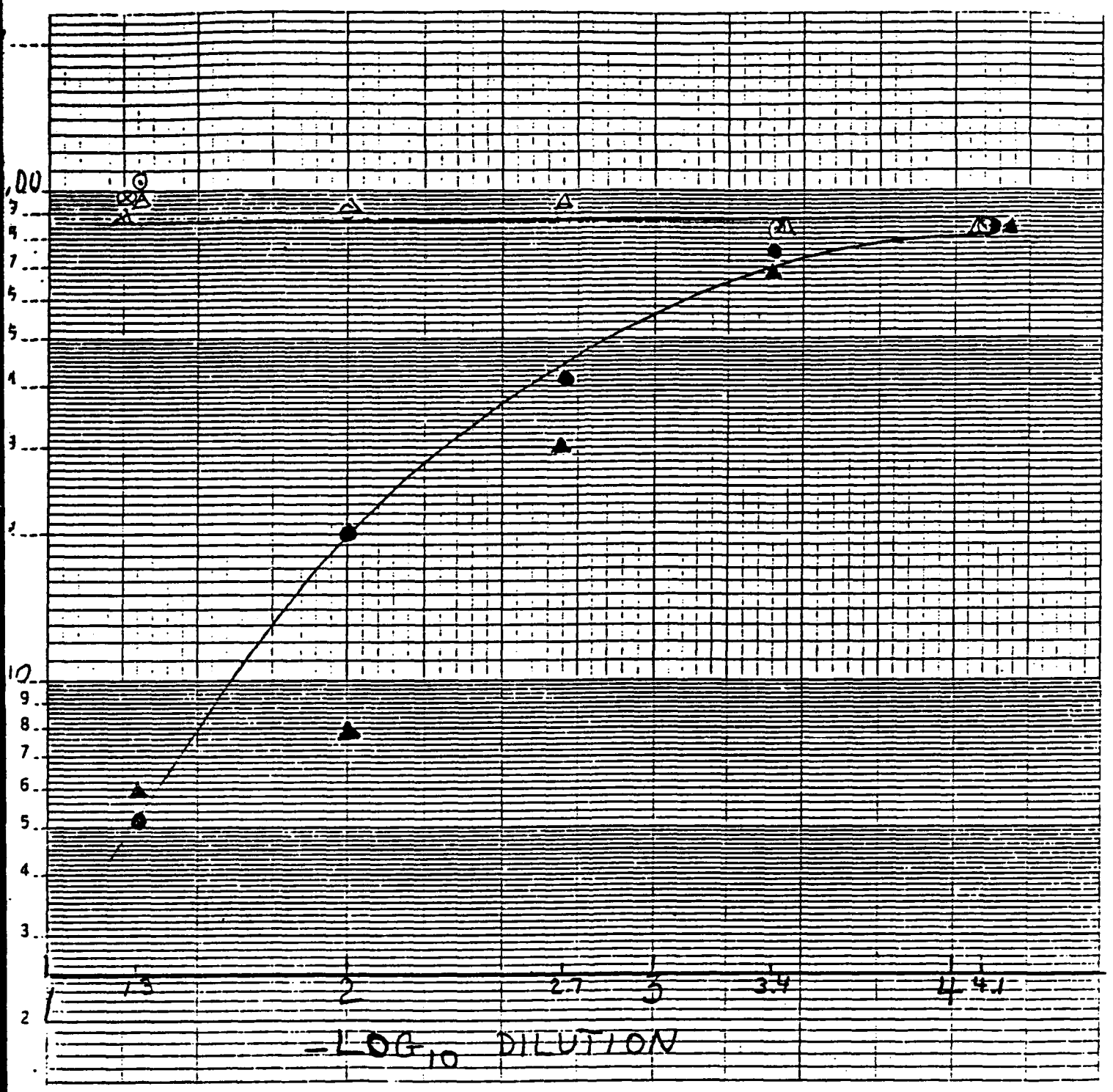


Figure 3. Competitive binding between rabbit sera against authentic or recombinant YF NS1 and biotinylated anti-NS1 Mab 1A5 or 8G4. Preimmune rabbit serum served as a control.
Biotinylated 1A5 vs: Rab ∝ auth NS1, ● ; Rab ∝ recom NS1, ○ ; preimmune serum, ⊗ .
Biotinylated 8G4 vs: Rab ∝ auth NS1, ▲ ; Rab ∝ recom NS1, △ ; preimmune serum, ⧋ .

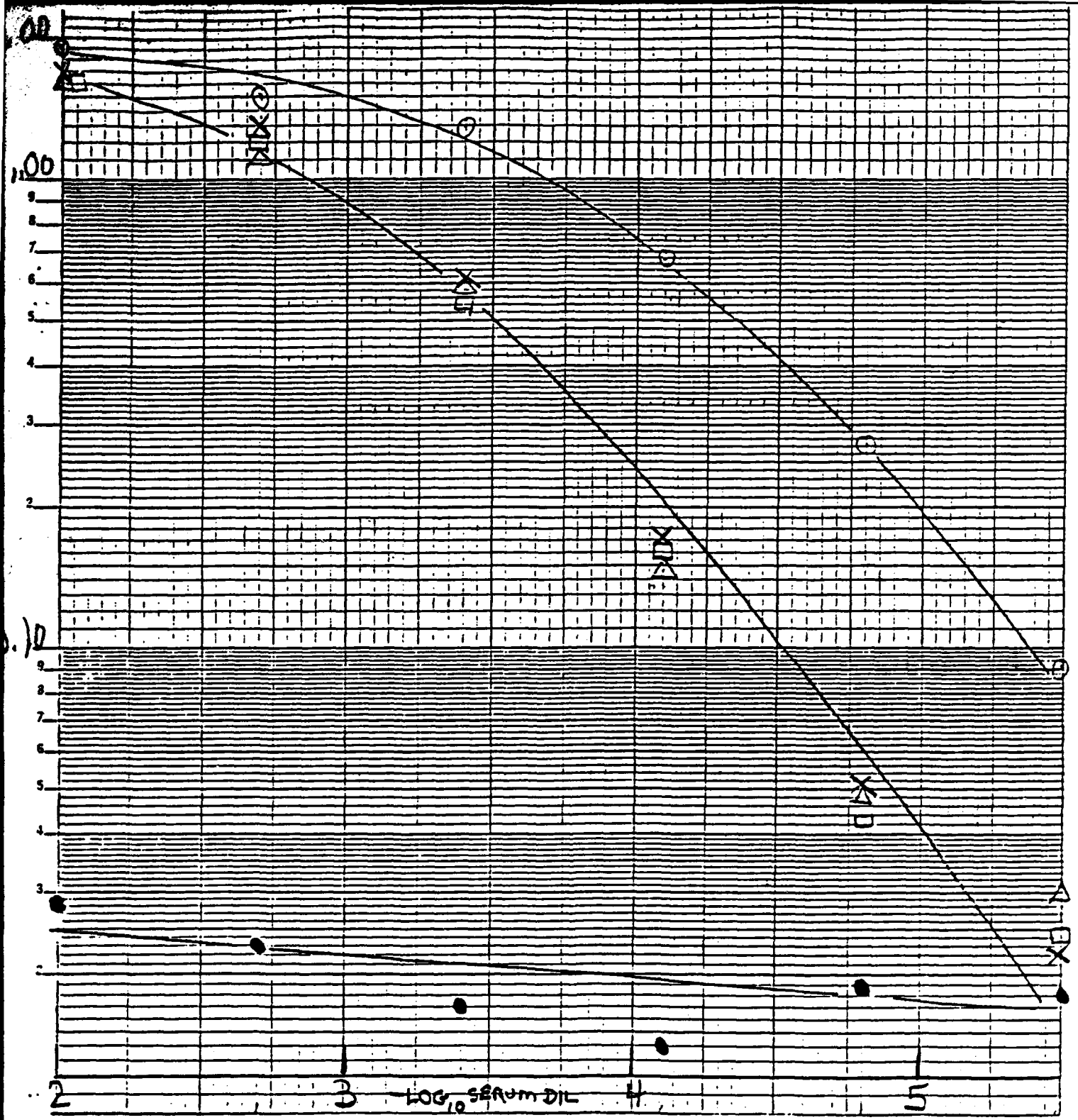


Figure 4. Relative binding avidities to authentic 17D NS1 of rabbit anti-auth NS1 ( O ) and consecutive bleeds from a rabbit immunized with recombinant 17D YF NS1 ( X , □ , △ ). Pre-immune rabbit serum ( ● ) served as a control.

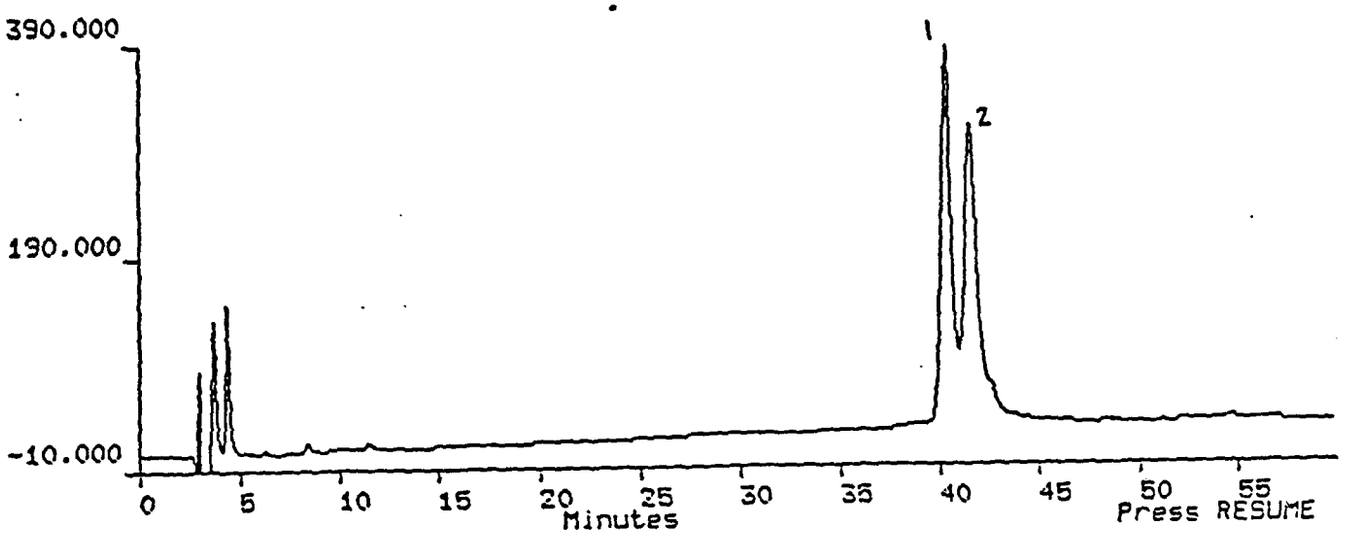


Figure 5. Fractionation of affinity-purified DEN 2 NS1. Approximately 10 umol *DEN 2 NS1 loaded onto Vydac C4* column and separated with a linear 30-80% Buffer B gradient at 1 ml/min. Buffer A 0.1% TFA. Buffer B 80% acetonitrile in Buffer A.
Peaks 1 and 2 reacted with anti-DEN 2 NS1 Mab (Western blot) and represent 46 kd (1) and 30 kd (2) moieties of DEN NS1.

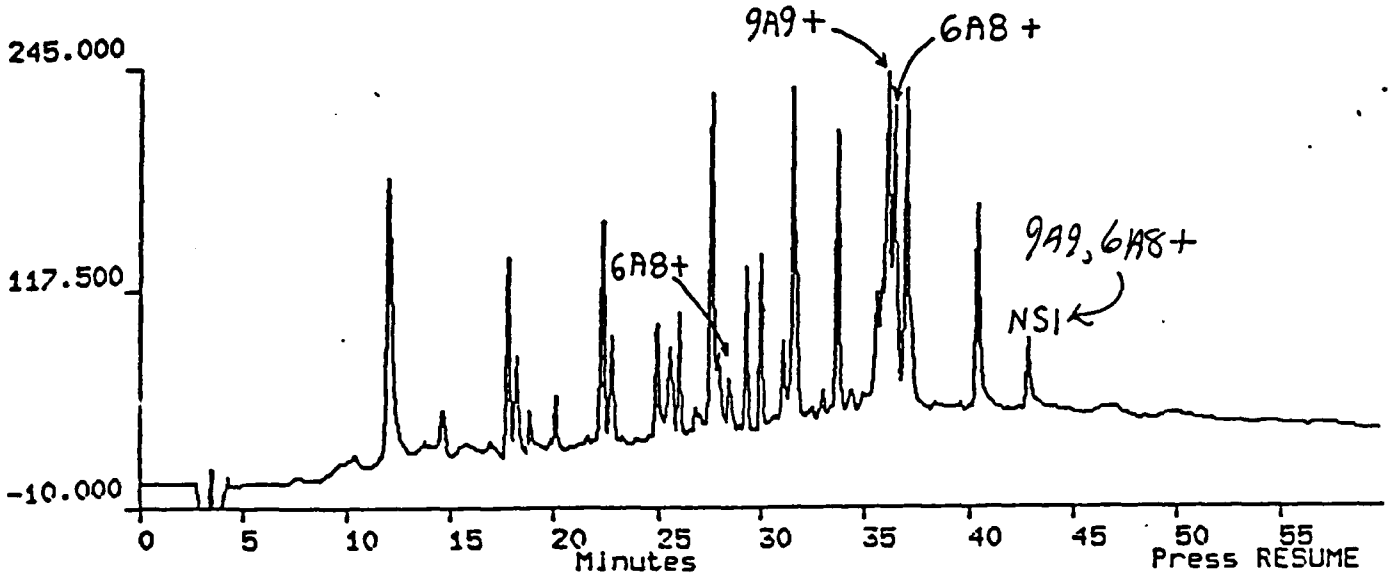


Figure 6. Fractionation and immunoassay of tryptic peptides derived from dithiothreitol-reduced DEN 2 NS1 (46 Kd). Ca 100 ug HPLC-purified NS1 in 50 mM ammonium bicarbonate pH 8.0 was digested with HPLC-purified trypsin (protein: enzyme 50:1) for 2 hours at 37°C and loaded onto a Vydac C18 column followed by separation with a 0-100% buffer B gradient. 1 ml/min and absorbance at 314 nm. Individual peptide peaks were lyophilized using a Savant Speed-Vac drier, reconstituted in phosphate buffered saline containing 0.1% Triton-X and tesetd by ELISA for reactivity (+) with "protective" anti-DEN NS1 Mab 65A8 or nonprotective Mab 9A9.

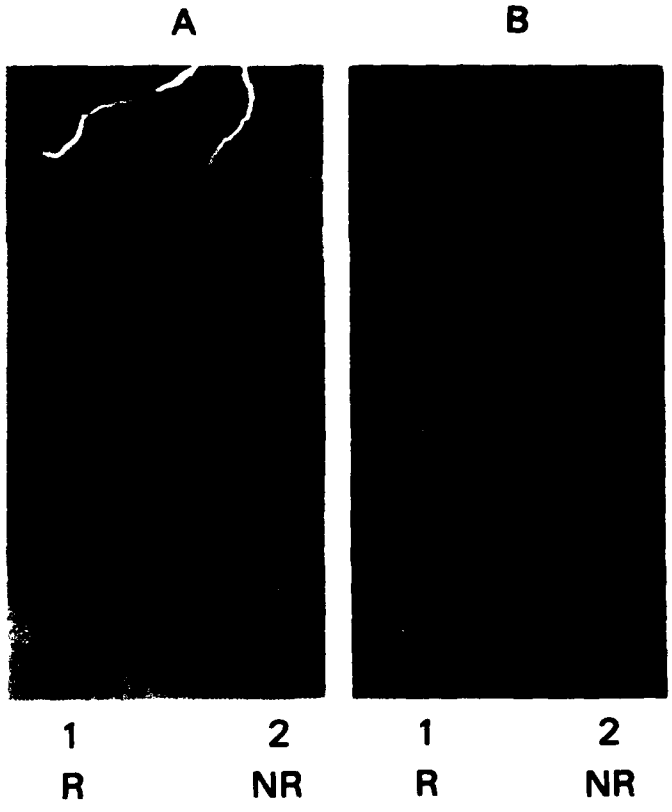


Figure 7. Acetylation of DEN 2 NS1 by $^{14}C$ iodacetamide. Panel A. Silver stain, 11% SDS polyacrylamide gel. Dithiothreitol-reduced (lane 1, "R") or unreduced (lane 2, "NR") NS1 treated with $^{14}C$ iodoacetamide. Panel B. Radioautogram of Panel A. Alkylation occurred only after reduction of NS1.

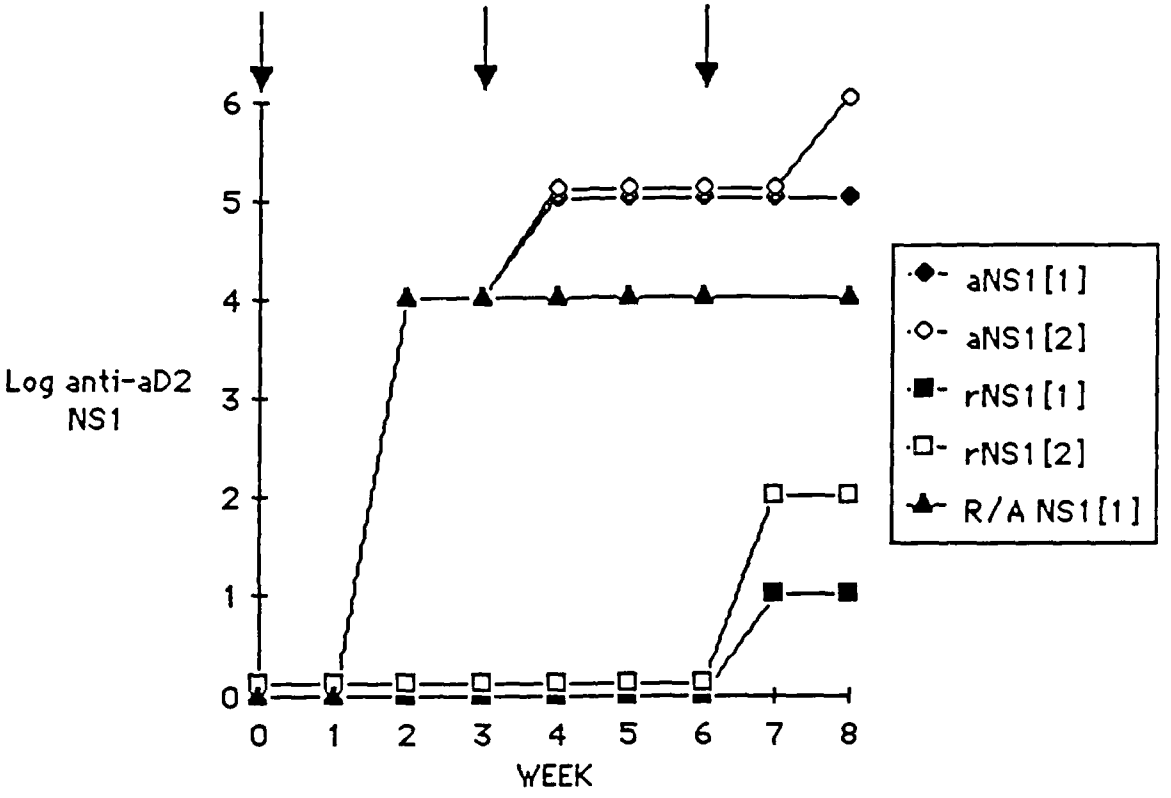


Figure 8. Antibody response (ELISA) against authentic (a) DEN 2 NS1 among rabbits immunized with aDen 2 NS1, recombinant (r) DEN 2 NS1 or reduced and alkylated (R/A) aNS1. Arrows indicate combined intradermal and subcutaneous injections of NS1 in complete Freund's adjuvant (prime) or incomplete Freund's adjuvant (boost). ca 25 ug prime; ca 10 ug booster doses were used.

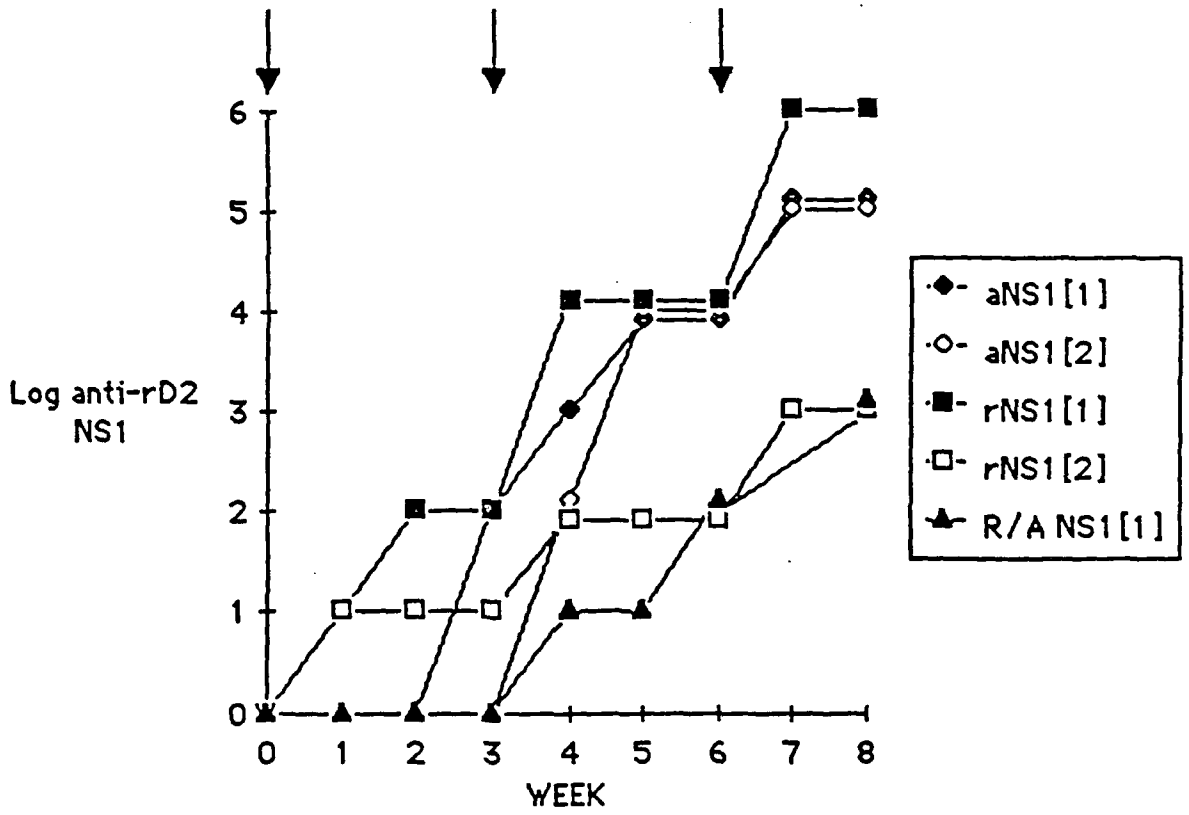


Figure 9. Antibody response (ELISA) against recombinant DEN 2 NS1 among rabbits immunized with authentic (a), recombinant (r), or reduced and alkylated (R/A) authentic DEN 2 NS1. Route, schedule and dosage as in Figure 8.

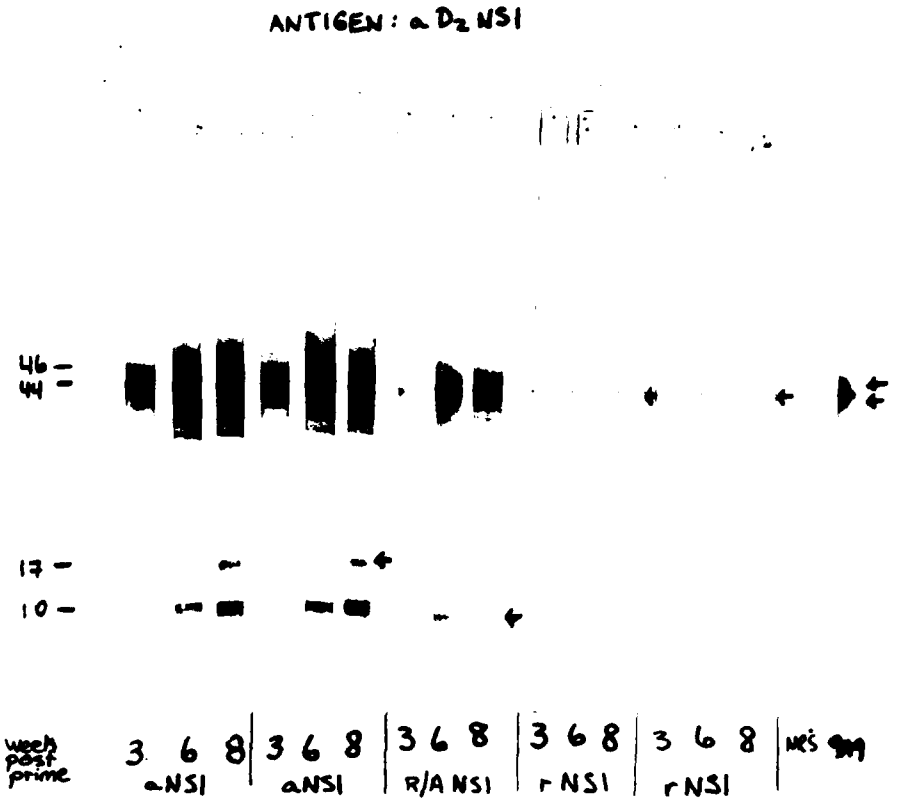


Figure 10. Western blot against authentic DEN 2 NS1. Rabbit sera drawn at 3,6, and 8 weeks following immunization with authentic (a), reduced and alkylated (R/A), or recombinant (r) DEN 2 NS1 (see Figs. 8 and 9). Preimmune serum (NRS) and anti-NS1 Mab 9A9 were controls.

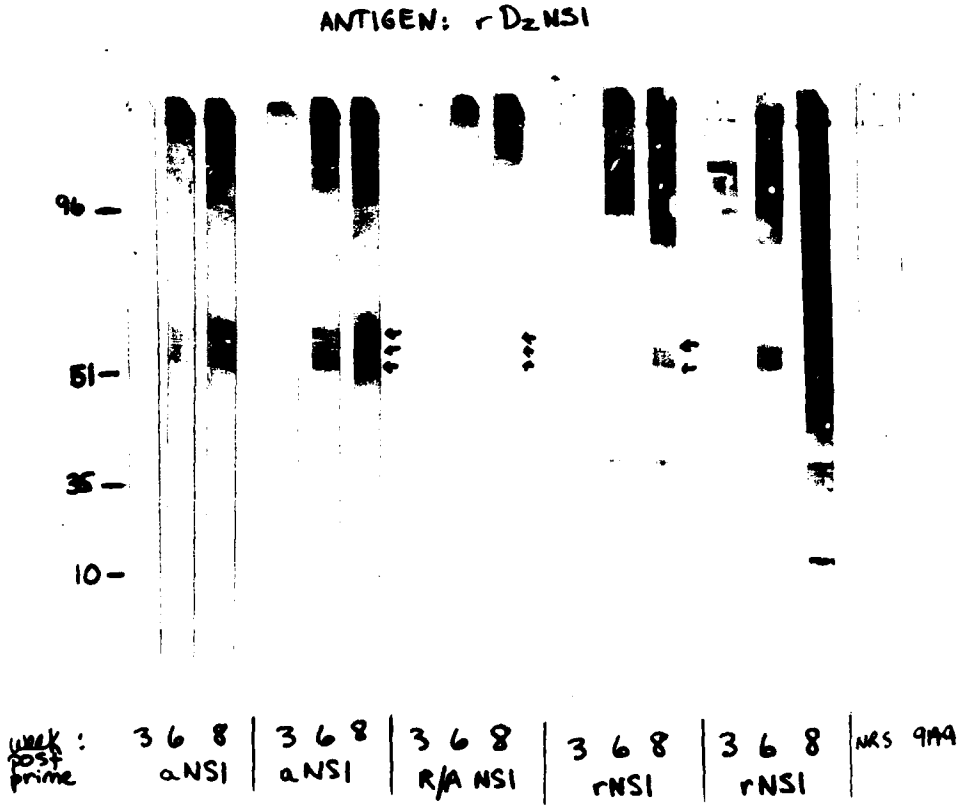


Figure 11. Antibody responses against recombinant (r) DEN 2 NS1 as in Fig. 10. Note failure of anti-aNS1 Mab 9A9 to react with rNS1.

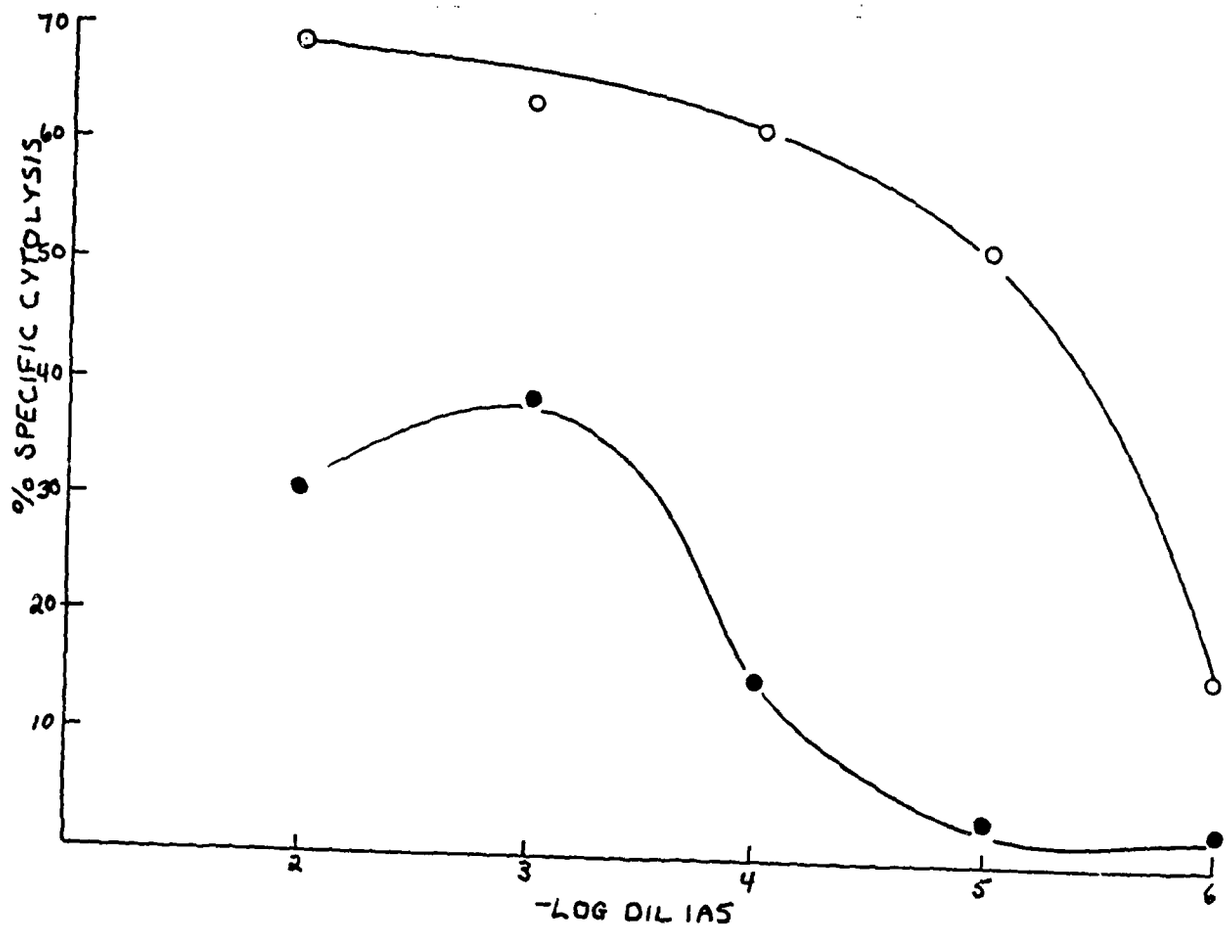


Figure 12. Cytolytic activity of serial dilutions of protective anti-NS1 Mab 1A5 in the presence of myeloma protein PC5 (O) or nonprotective, nonlytic anti-NS1 Mab 2D10 (●).

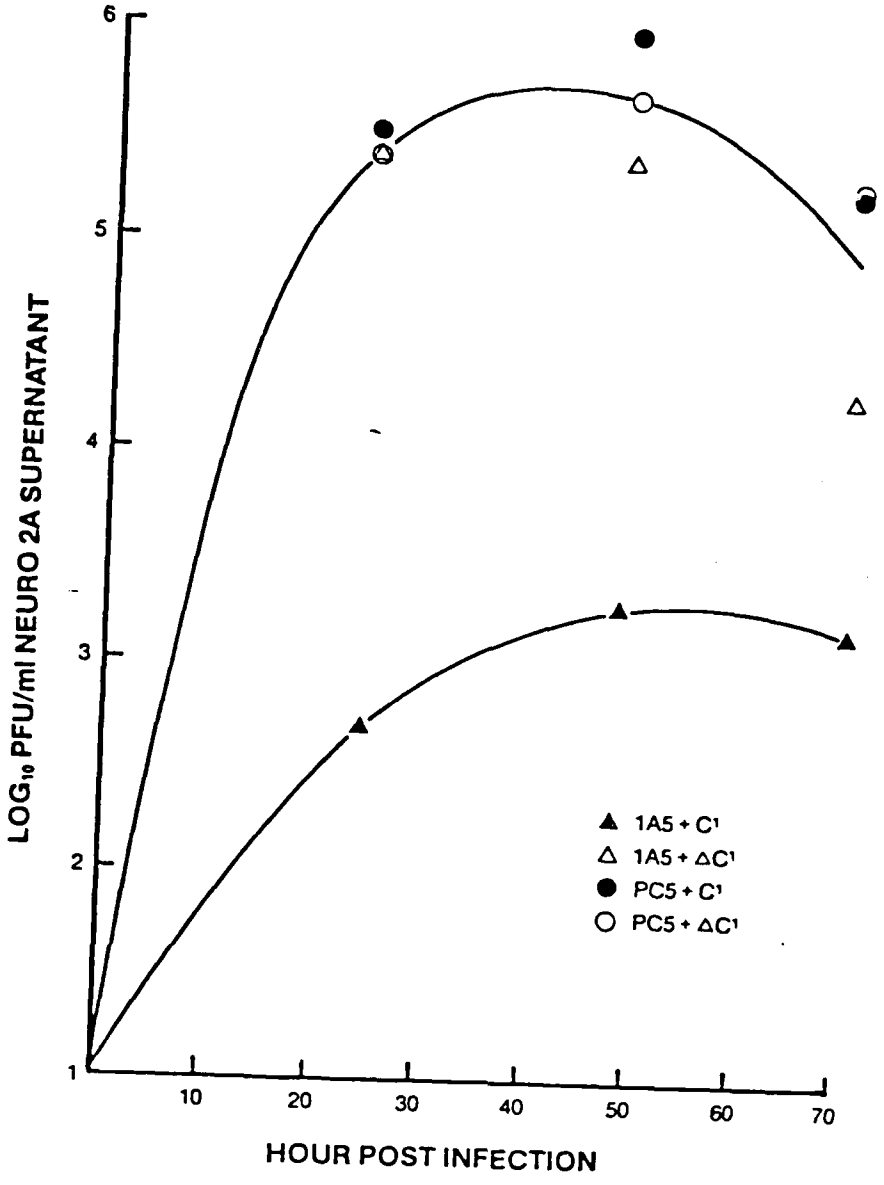


Figure 13. Effect of antibodies on infection of neuroblastoma cells. Neuro 2a cell monolayers grown in 22 mm plastic Costar cluster plates were infected with 17D YF at 37°C. After incubation for 60 minutes, residual virus was removed by multiple washes and the media replaced with MEM-10% FCS containing anti-NS1 monoclonal antibody 1A5 at a final dilution of $10^{-2}$ and active ( ▲ ) or heat-inactivated ( △ ) rabbit complement added to a final dilution of 1/20. Myeloma protein PC-5, added with ( ● ) and without ( ○ ) active complement, served as a control. At 24 hour intervals, supernatant media was sampled for infectious virus in a Vero cell plaque assay and active or heat-inactivated complement was replenished in remaining wells to a final dilution of 1/20.